ALKANE DISTRIBUTION AND CHEMOTAXONOMY OF TREE FERNS (CYATHEACEAE)

by

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ABSTRACT

A gas chromatographic study was made of the total n-alkane content of several species of Central American tree ferns (Cyatheaceae). Some species of commonly occuring North American ferns were also studied for comparison. The species studied exhibited the expected n-alkane distribution pattern. Attempted taxonomic correlations had very limited success. The possible existence of a comprehensive taxonomic classification utilizing n-alkanes should be studied more fully when larger samples are available.

INTRODUCTION

The exterior covering of all higher plants consists primarily of waxy esters, hydrocarbons, primary alcohols, aldehydes, and fatty acids. The hydrocarbons are normally n-alkanes containing 21 to 35 carbon atoms. Odd-numbered alkane chains are normally more abundant than the even numbered chains with usually one member of the C_{27}-C_{33} group predominating.[1,2]

The taxonomic importance of the n-alkanes has been suggested by Eglinton.[3,4] The results of Eglinton's correlations and those of other investigators involving the n-alkane fraction of plant waxes has been reviewed.[5,6]

The alkane distrubution in ferns has been examined only to a limited extent.[7] It is the purpose of this investigation to study the n-alkane distribution patterns of several species of Central American tree ferns. Furthermore, attempts to arrive at a possible taxomomic scheme will be made.

HISTORICAL

A. The Plant Cuticle

The outer surfaces of all higher plants are covered with a protective water-repellent material called the plant cuticle. The cuticle is composed of two distinct layers: (a) the cutin (e.g. cross-linked hydroxy acids) lying innermost to the epidermal cells, and (b) a waxy outer layer exposed to the atmosphere.[5] The chemical composition of the waxy outer covering is summarized in Table 1.

The physical nature of the epicuticular waxes has been studied.[8,9,10] The possible importance of this covering has been investigated.[11,12]

Numerous reviews have appeared concerning the physical, chemical, biochemical, and physiological aspects of the cuticle.[1,2,13,14,15]

B. The Hydrocarbon Fraction of the Cuticle Wax

Chibnall[16,17] first demonstrated conclusively the existence of long chained odd-numbered carbon n-alkanes as major components of plant hydrocarbons. Evidence for the possible presence of even-numbered alkanes was not reported until 1953 by Schuette[18] and positive identification was not made until 1955 by Wanless.[19]

The existence of iso-alkanes,[20] anteiso-alkanes,[21]

Table 1

Major Components of Leaf Waxes[5]

Type	Range	Frequency
Alkanes	Normal : odd C_{21}-C_{37}	common, especially C_{29} and C_{31}
	Normal : even C_{20}-C_{34}	common minor components
	Branched : C_{27}-C_{33}	infrequent
Alcohols (usually as esters)	Primary : even C_{22}-C_{32}	common
	Primary : odd C_{25}-C_{31}	infrequent
	Secondary : odd C_{21}-C_{33}	common
	Diols and ketols	rare
	Terpene alcohols	infrequent
Aldehydes (as polymers)	Normals : C_{24}-C_{34}	rare
Ketones	Di-n-ketones	rare
Acids (usually as esters)	Normal : even C_{12}-C_{34}	common
	Normal : odd C_{15}-C_{33}	(?)
	Ketoacids	rare
	Dibasic acids	rare
Esters	Between n-acids and primary and secondary alcohols	common
	Estolides of Hydroxy-acids	infrequent

cyclic hydrocarbons,[22,23] olefinic and acetylenic compounds[24,25] have been reported as minor components of plant waxes.

Eglinton[3,5] reports, the following generalizations concerning the distrubution of alkanes in plant waxes:

"a. Alkanes of carbon number less than C_{25} and more than C_{35} are rarely present to any appreciable extent.

b. The content of odd-numbered alkanes is usually greater than that of the even numbered alkanes by a factor of more than ten, but small amounts of the latter are almost invariably present.

c. The major constitutent is often...(either)... C_{27}, C_{29}, C_{31}, or C_{33} n-alkanes.

d. A high proportion of iso-alkane is rare, and even trace quantities are uncommon.

e. For the major odd-numbered constituents (e.g. C_{31} and C_{33}) of any leaf wax there are indications of a parallelism in the iso-to-normal hydrocarbon ratio."

C. <u>The Alkane Fraction and Chemotaxonomy</u>

Eglinton[3] has suggested that the distribution of n-alkanes could best be used for taxonomic identification for the following reasons:

a. The alkanes are not primary metabolic compounds but are the by-products of the normal metabolic proc-

ess.

b. The alkanes represent stable biological markers and are insensitive to extraction procedures and methods of analysis.

c. The alkanes are easily isolated and identified.

d. The alkanes of the plant cuticle wax tend to be insensitive to seasonal variations.

Mirov has used the alkanes of the genus Pinus to distinguish between P. jeffrey and P. ponderosa.[5] Isolation of the single alkane undecane from P. refexa and P. monticola permitted him to form a rather limited classification of these two species.[26]

Eglinton found a rather useful relationship between the characteristic alkanes of the sub-family Sempervivoideae.[3,5] These studies resulted in the generalizations previously mentioned.

Aplin[27] and Mold[28] have studied the diterpene hydrocarbons of Podocarpaceae and varities of tobacco, respectively.

The above reports and those of other investigators have lead Eglinton to conclude that it is possible to form a relible taxonomic scheme involving the alkane distribution in biologically related species. However, there has only been limited use of the alkanes for this purpose. The most com-

plete and comprehensive study being that of Eglinton which tends to indicate that the alkane distribution may be sufficient to detect differences in closely related species but is not discriminating enough to be helpful in studying related genera.[5]

Douglas[6] states that the usefulness of chemotaxonomy by alkanes is unlikely to develop completely until analytical procedures are improved and the mode of biosynthesis of the alkanes can be determined.

Berti[29] cites only three references involving the alkane distribution in ferns, none of which are tree ferns. Taxonomic patterns involving ferns and their alkane fraction have not been reported.

DISCUSSION OF RESULTS

A. Generalizations

Fifteen species of ferns were studied, eleven species of which were tree ferns (Cyntheaceae). The four species of locally occuring ferns were studied as a comparison and to test experimental procedure.

Table 2 lists the identification of the species used and the code numbers assigned to the species. The use of code numbers will be used on all charts and tables.

The alkane fraction examined represents the total alkane content of the species studied. It is assumed that this takes into account the epicuticular as well as that fraction of alkanes present within the cytoplasam. Several investigators have proviously determined the existence of alkanes within the cytoplasam.[33,34] Since the samples were in a state of extreme dryness the method used (see experimental section) was considered to be the best means of extraction.

The amount of wax from the fronds of the tree ferns is generally the same as that present in those ferns which grow locally. However, the apparent amount of alkanes in the wax of the tropical ferns was considerably less than that recovered from the local species studied. (see Table 3) Differences in the amount of alkanes may be due to the dif-

Table 2

Identification of Fern Species Studied

Code Number	Systematic Name
003	Metaxya rostrota (Hbk.) Presl.
016	Spheropteris elongata (Hook) Tryon
018	Lophosoria (Sp)
020	Cenemedaria (Sp)
025	Dicksonia (Sp) Not Cyatheaceae but Pteridaceae
029	Nephelea (Sp)
030	Cyathea (Sp)
034	Spheropteris Brunei (Christ) Tryon
036	Cyathea onusta (Christ)
037	Cyathea Tuerckheimii Maxon
041	Cyathea divergens-Kze.
Xmas	Polystichum acrostichoides (Michaux) Schott
D.marg.	Dryopteris marginalis (L.) Gray
D.int.	Dryopteris intermedia (Willd) Gray
P.vir.	Polypodium virginianum L.

Table 3

Weights and Percentages of Wax Fractions Recovered[1]

Sample	Weight Fronds	Weight Wax Collected	Weight Alkane Fraction	% Wax Collected	% Alkane Fraction *
003	20.3836	0.5618	-	2.75	-
016	25.0864	1.1667	0.0504	4.65	4.32
018	46.[a]	-	0.0184	-	-
020	34.1958	0.6602	0.0126	1.92	1.99
025	26.6165	0.1943	0.0192	0.83	9.89
029	40.81[a]	-	0.0139	-	-
030	23.7221	0.6105	0.0547	2.57	9.0
034	36.8684	0.7976	0.0767	2.16	9.6
036	22.2086	0.2781	-	1.2	-
037	31.5076	0.3501	0.0255	1.1	7.1
041	52.17[a]	-	0.0187	-	-
Xmas	-	-	-	-	-
D.marg.	18.8330	0.5531	-	2.9	-
D.int.	19.6491	0.3491	-	1.7	-
P.vir.	25.4542	0.4207	-	1.5	-

1 All weights are in grams.
a These measurements are not exact.
* % alkane fraction is computed on the basis of the % wax collected..

ferent methods by which these two categories were dried, or it may represent a true biological difference which is a function of the environmental characteristics of the localities from which these samples were gathered.

Futher differences in the alkane fractions of these species lie in the amount of unidentified material that was present after column chromatography. The tropical ferns show considerable material other than n-alkanes, evident from the gas chromatograms and infrared spectra. The possible nature of this material is proposed to be that of triterpenoid ethers as reported by Eglinton.[3,5] This observation was based on absorption at 1120 $cm.^{-1}$ due to carbon-oxygen stretching.

Gas chromatograms of the tree fern samples were complicated by the presence of several unidentified peaks, which in several cases constituted a major portion of the samples. The results obtained for these samples are therefore to be considered questionable, since the n-alkane peaks are often hidden or appear as shoulders to these unidentified peaks. In most cases it was found that the position of non-alkane peaks could be shifted by changing the liquid phase. The various Kovat Retention Index Numbers for these peaks are given in Table 4, as a function of the liquid phase used. The gas chromatograms of locally occuring fern alkane fractions show little if any unidentified material.

Table 4

Kovat Retention Index Numbers For Major Unidentifiable Peaks

Sample	SE-30	Apiezon-L
003	3150	-
	-	3150
	-	3050
016	2800	2750
	3150	3250
018	3050	3150
	3150	3250
020	2800	2740
025	-	-
029	3150	3250
	2450	2500
030	2800	2750
	3150	3250
034	2800	2750
	-	3150
	3150	3250
	3250	3350
037	2800	2740
	3160	3250
036	-	3200
041	2800	2750
	2950	-
Xmas	-	-
D.marg	3050	3150
	1850	-
	1980	-
	-	3250
D.int	3040	-
P.vir	3150	3250

Examples of gas chromatograms of the tropical fern (Cyathea Tuerckheimii) and the local fern (Polystichum acrostichoides) are given for comparison in Figures 1 and 2. The shifting position of unidentified material as a result of changing the liquid phase can be seen in Figure 2. It should be noted that a peak occuring at Kovat Index 2800 on SE-30 appears as 2750 on Apiezon-L.

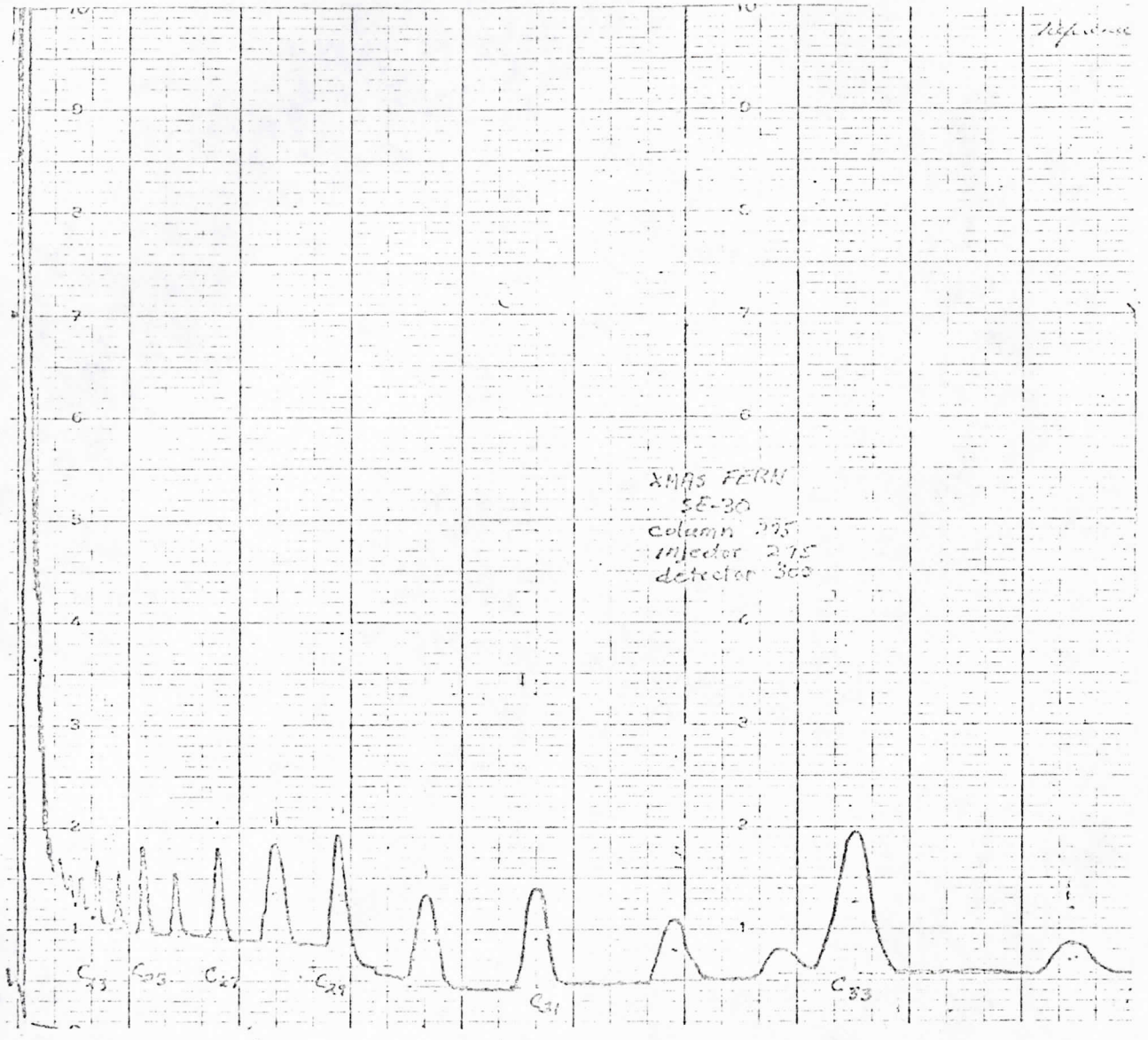
Infrared spectra were run on several of the samples. The spectra indicates the presence of material other than alkanes in the samples of tropical ferns. Eglinton[3] has assigned the absorption 1,120 $cm.^{-1}$ as that belonging to triterpenoid ethers. Figure 3 illustrates the spectra obtained from a typical tree fern (029) sample and that of a local fern (Polystichum acrostichoides).

Table 5 is a listing of various absorptions observed for those samples of which infrared spectra were recorded.

B. Alkane Distribution and Chemotaxonomy

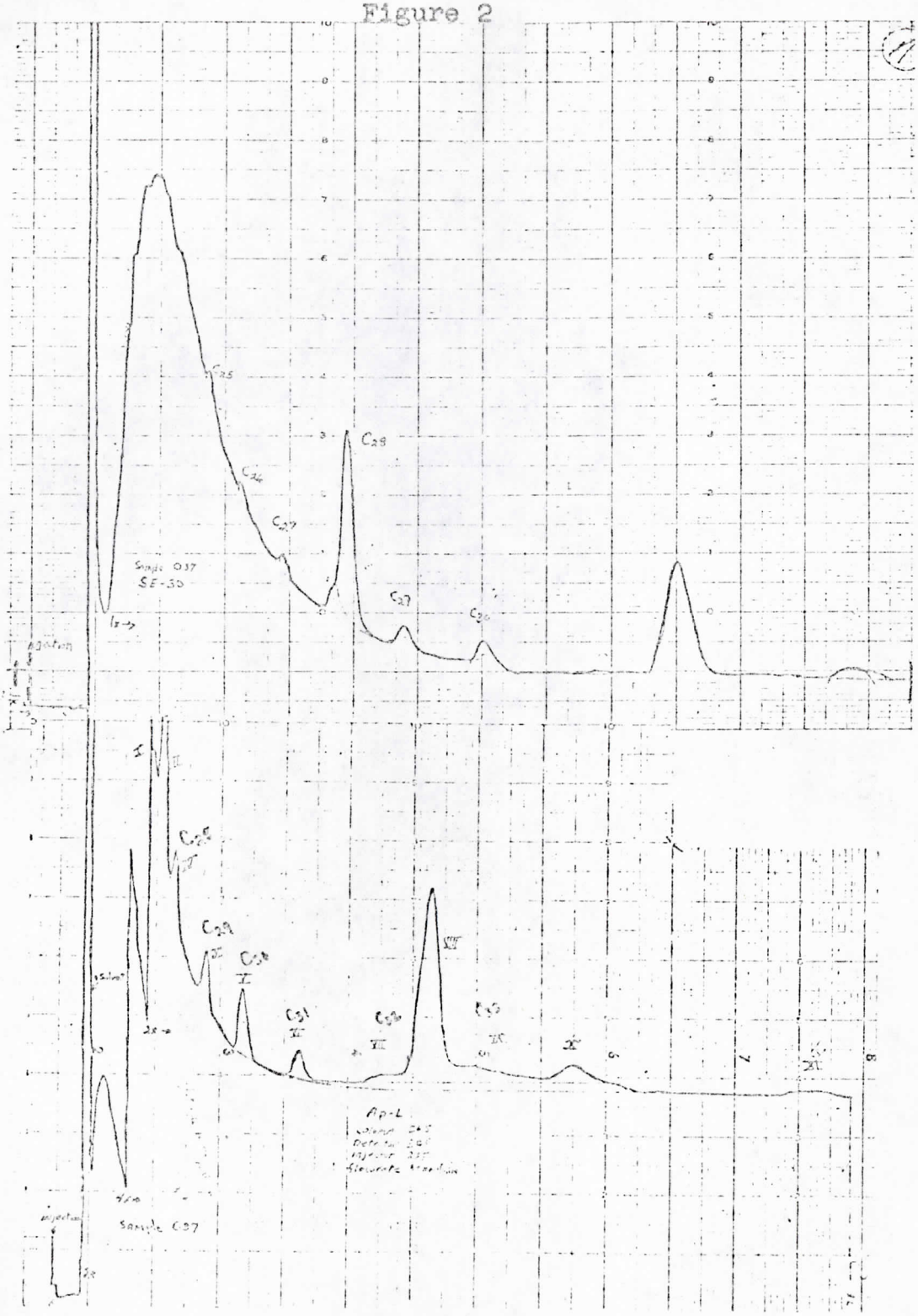
All the samples studied show n-alkane distributions that correspond to the generalizations proposed by Eglinton[3,5] (see page 4) Due to presence of unidentified materials in the alkane fraction of the tropical ferns, several of these species are only assumed to have a complete alkane distribution from C_{25} to C_{33}. Often only one or

Figure 1



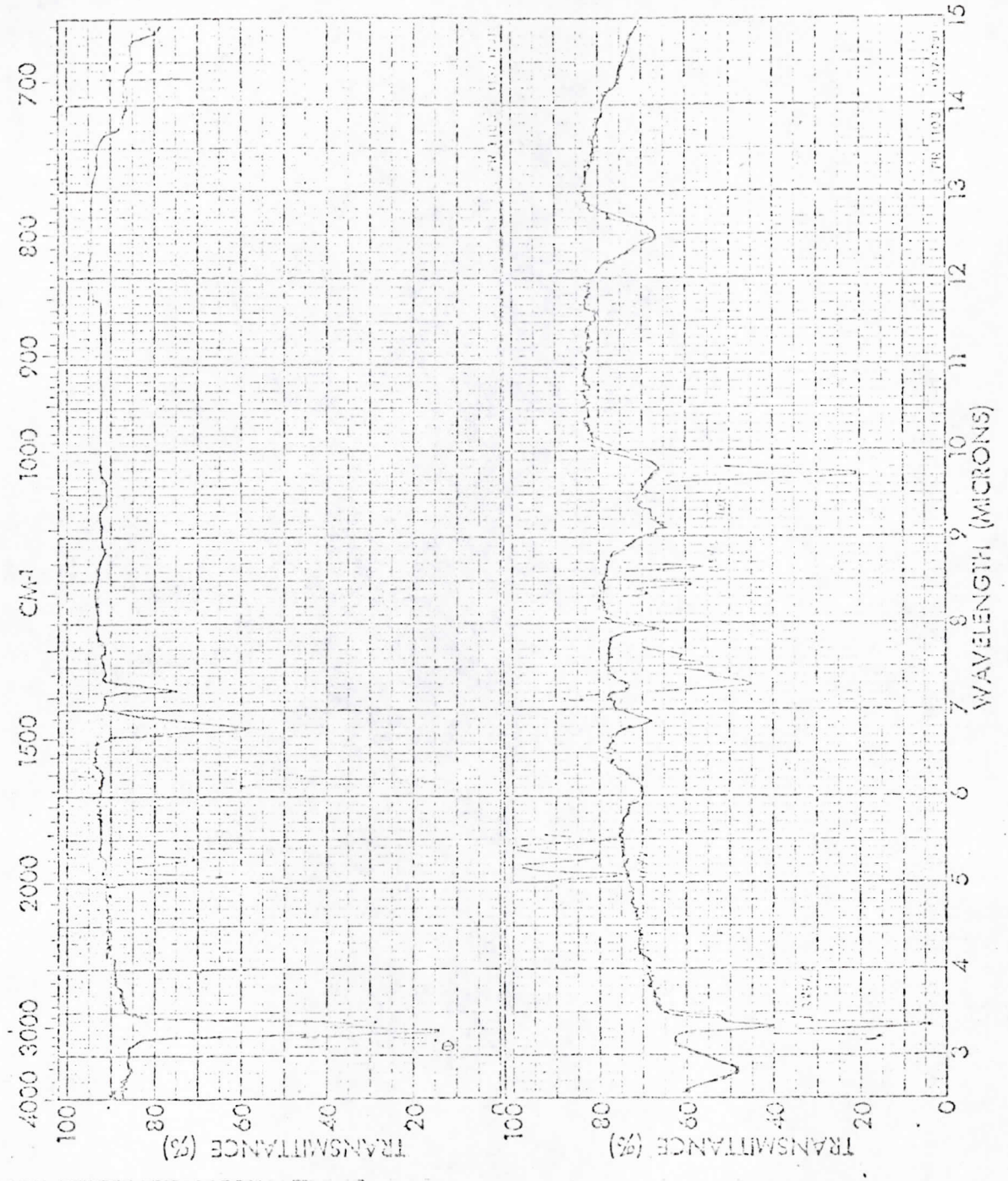
Gas Chromatogram of Alkane Fraction from Locally Occuring Fern, Polystichum acrostichoikes.

Figure 2



Gas Chromatograms of Alkane Fraction from Tropical Fern, Cyathea Tuerckheimii. Change in liquid phase illustrated: SE-30 top: and Apiezon-L, bottom.

Figure 3



Infrared Spectra of Local Fern (Polystichum acrostichoides); top and Tropical Fern (029); bottom.

Table 5

Infrared Absorptions for Several Alkane Fractions

Sample	Absorption, $cm.^{-1}$	
Xmas-crude wax	1750	s
	1260	w
Xmas-alkane fraction	1775	vs
	1175	s
018	1120-1075	b
029	1600-1700	b
	1250	s
	1100-1000	b
020	1700	
	1650	
	1600	
	1150	
034	1750	
	1650-1675	

s-strong
w-weak
b-broad
vs very strong

two peaks could be identified. It is unlikely that these are the only alkanes present. With further sampling and larger sample sizes, as well as improved technique, these samples should reveal the normal alkane distribution patterns.

Several species showed the predominance of just one alkane while others showed an apparently equal quantity of two or more alkanes. The species studied can be divided into seven classes based on their distribution patterns. These include: Those species that contain only one alkane; C_{29}, C_{30}, C_{31}, or C_{33}; and those species which contain predominately two alkanes: C_{27}-C_{29}, C_{29}-C_{33}, or C_{31}-C_{33}. The species which correspond to each of these classes are listed in Table 6.

The complete alkane distributions measured for each specie appear in Table 7.

Locally occuring and biologically related species of Dryopteris intermedia and D. marginalis show considerable quantities of C_{29}, which differeniates them from the other two locally occuring species of Polypodium virginianum and Polystichum acrostichoides. The latter species show C_{33} as their major component, and are in themselves differeniated by the ratio of C_{33} to other odd-numbered alkanes present. The alkane distribution for these species are presented in histogram form in Figure 4.

The amount of data availiable for the tropical

Table 6

Classification of Species According to Predominate Alkanes

C_{29}

D.Marg.

D.int.

020

029

034

041

C_{30}

037 Results are questioned on bases of Eglinton's correlations

C_{31}

003

C_{33}

P.vir.

Xmas

C_{27}-C_{29}

036

025

C_{29}-C_{33}

030

016

C_{31}-C_{33}

018

Table 7

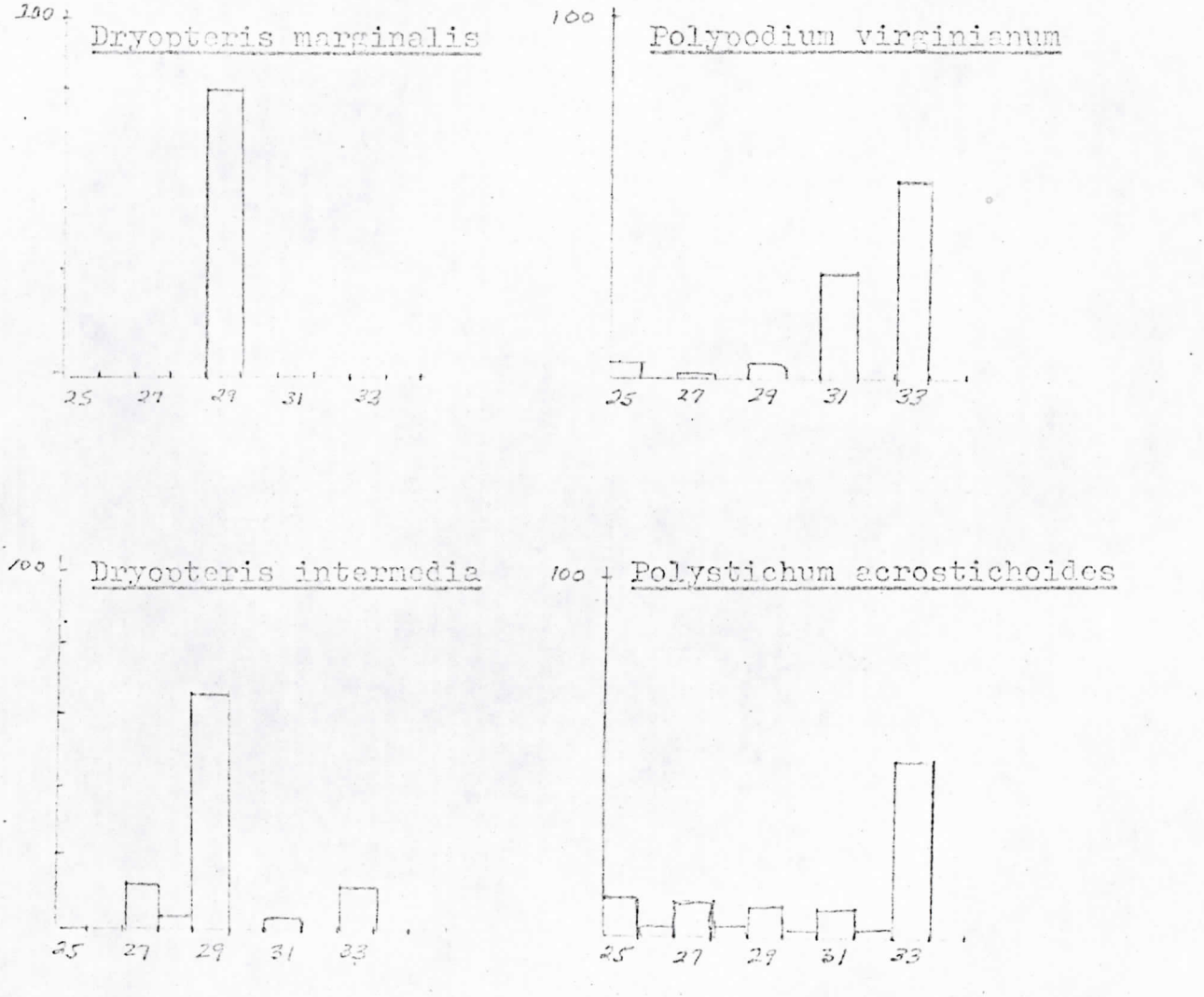
Alkane Distribution Patterns In Fern Species Studied

Code Number	Alkanes 23	24	25	26	27	28	29	30	31	32	33
003	0	0	-	-	-	-	10	8	65	-	16
016	0	0	34	0	0	0	31	0	0	0	35
018	0	0	0	0	0	0	m	0	M	0	m
020	0	0	0	0	m	-	M	m	m	0	0
025	0	0	0	0	m	-	M	0	0	0	0
029	-	-	-	-	12	20	29	17	23	-	0
030	0	0	0	0	-	0	56	-	-	-	43
034	-	-	-	-	-	-	64	7	23	-	-
036	0	0	0	0	47	29	58	5*	10*	0	0
037	0	0	0	0	0	17	25	36	21	0	-
041	-	-	-	1.3	4.4	13	28	8.8	4.4	-	0
Xmas	3.5	2.5	7	4.5	8	5	8.6	3.5	8	4	50
D.marg.	-	-	-	-	-	-	80'	0	0	0	0
D.int.	-	-	-	-	13	6	65	--	7	-	12
P.vir.	0	0	6	-	3	-	7	-	30	-	55

0 not seen
- trace
* less than
' greater than
M major component, approximation
m minor component, approximation

Figure 4

Alkane Distribution in Local Ferns

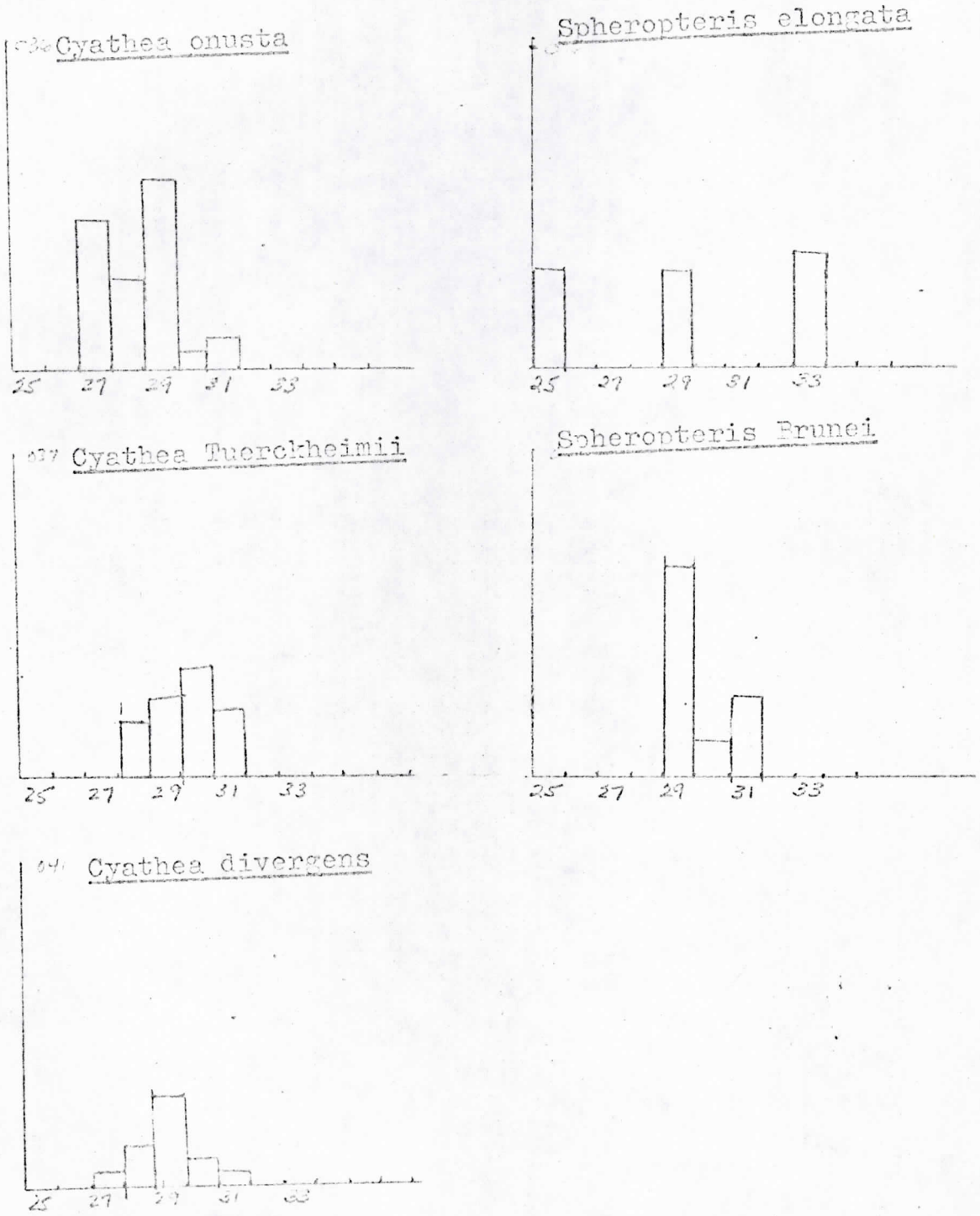


Assigned carbon numbers (abssissa) vs. Relative Percentage of Alkane Observed.

fern does not permit analysis of their taxonomic relationship. However, it is noted that those species belonging to the genera Cyathea show a predominance of C_{29} and a regularily decreasing quantity of alkanes of carbon numbers above and below C_{29}. While those species of Speropteris show irregular quantities of alkanes above and below C_{29}. This data is presented in Histogram form in Figure 5.

Figure 5

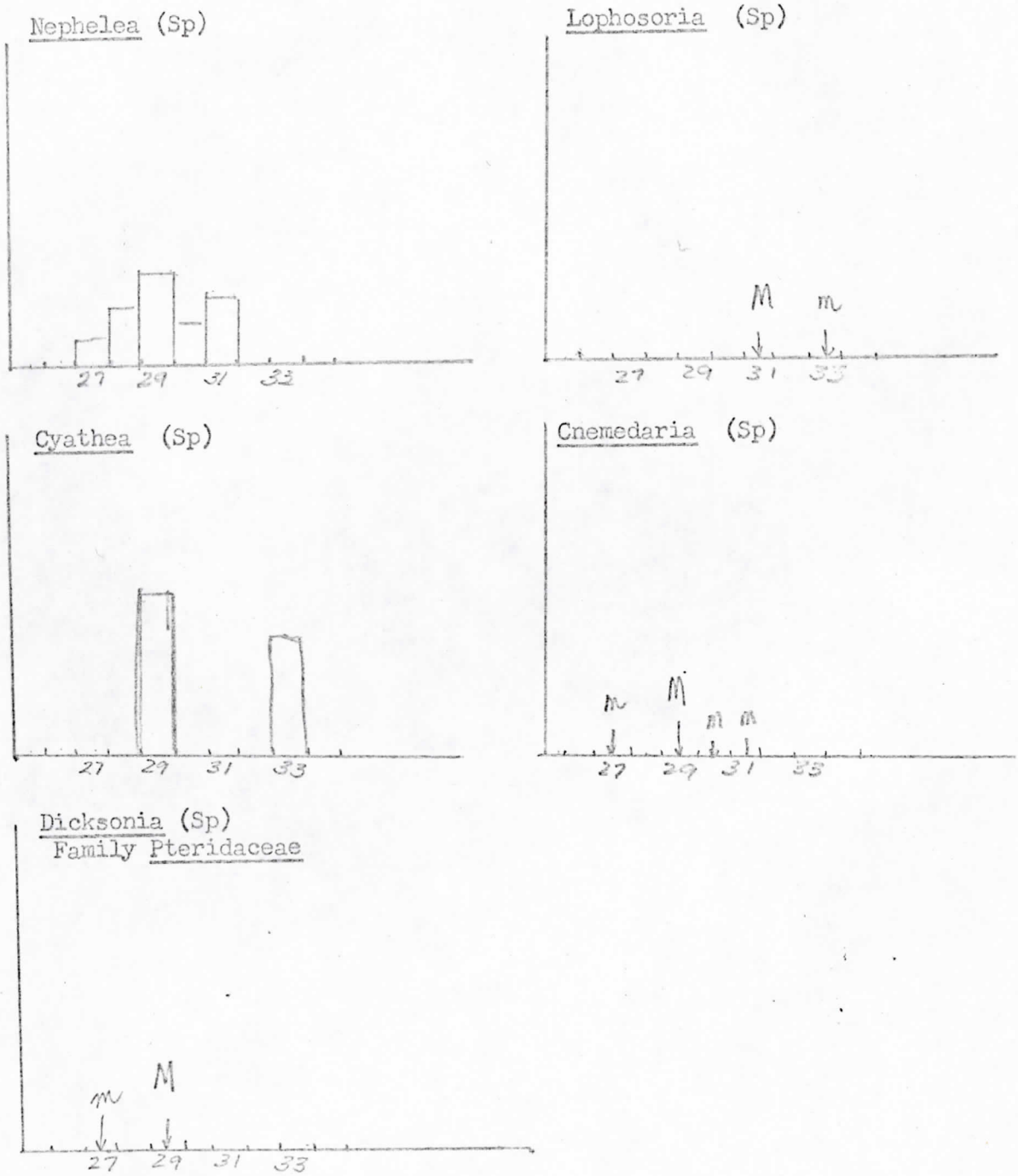
Alkane Distribution in Tropical Ferns



See figure 4 for explanation of scale.

Figure 5 (con't)

Alkane Distribution in Tropical Ferns



See Figure 4 for explanation of scale.

CONCLUSIONS

The ferns studied exhibit the normal n-alkane distribution with the wax content averaging approximatly 1-3% of the weight of the frond and the alkane content averagi 7-10% of the wax. The tropical ferns studied exhibit considerable material which is not removed by column chromatography and this may represent a chemical difference in the waxes recovered from these samples and thcse of the locally occuring species.

The results that were obtained from the study of the Dryopteris species and those of the Polystichum acrostichoides and Polypodium virginianum indicates that these species can be differentiated by their alkane distribution patterns.

Further collection and samplying is necessary to expand the study of the tree ferns. It is belived that a change in the procedure requiring pre-treatment of the wax samples with concentrated sulfuric acid prior to column chromatography would be helpful in removing long chain ethers.

EXPERIMENTAL

A. Collection and Handling of Samples

Samples of Central American tree ferns were collected in Costa Rica during the month of May, 1971. The ferns were cut fresh and artifically dried.

Samples of locally occuring ferns were collected during the months of August and September, 1971. These samples were dried by allowing them to sit for a period of two weeks at room temperature.

Identification of the tropical ferns was preformed by R. Tryon, Harvard University, Cambridge, Mass. and those of the local ferns by I. W. Carpenter, Department of Biology, Appalachian State University, Boone, North Carolina.

The dried fronds were prepared for extraction by masceration in a Waring blender.

B. Isolation of the Total Wax

The total waxes were removed by soaking the macerated fronds in enough purified n-hexane to just cover the bulk of the sample. The weight of the ground fronds was recorded prior to extraction, this data appears in Table 3.

Each sample was extracted six times, total extraction time being 36 to 62 hours. The combined extract from each sample was filtered and allowed to air evaporate.

After evaporation of the solvent the residue was combined with a weighed portion of alumina. The weight increase of the alumina was recorded as the total wax extracted. (see Table 3)

C. Isolation of the Alkane Fraction

The wax-alumina mixtures from the above procedure were placed at the top of previously prepared alumina columns and the columns eluded with n-hexane. The total volume of the n-hexane collected, in preweighed collection bottles, was between 75 and 100 milliliters.

The n-hexane collected was then allowed to air evaporate and the weight increase of the collection bottles was recorded as the weight of the alkane fraction. (see Table 3)

The resulting residue was diluted to a volume of 0.5 milliliters using n-hexane and this solution used for gas chromatographic analysis.

Columns of alumina were constructed using 100-ml. burets, fitted with Teflon stopcocks. The total length of the columns ranged from 25 to 30 centimeters. The alumina used was chromatography grade, 80-300 mesh, activated alumina purchased from Matheson, Coleman, and Bell, Inc. (AX 612)

A Varian Aerograph 1520 gas chromatograph fitted with thermal detectors, a Honeywell model ElectroniK 15 re-

corder and a column (10feet by 0.25 inch, aluminum) packed with Chromasorb W coated with one of the following liquid phases: 1% Apiezon-L or 1% SE-30 was used. Coatings were applied by the funnel coating method.[30]

In general the gas chromatograms were recorded under isothermal conditions using helium as the carrier gas. The columns were run at a temperature of 280, detectors at 310, and injectors at 275 degrees centigrade.

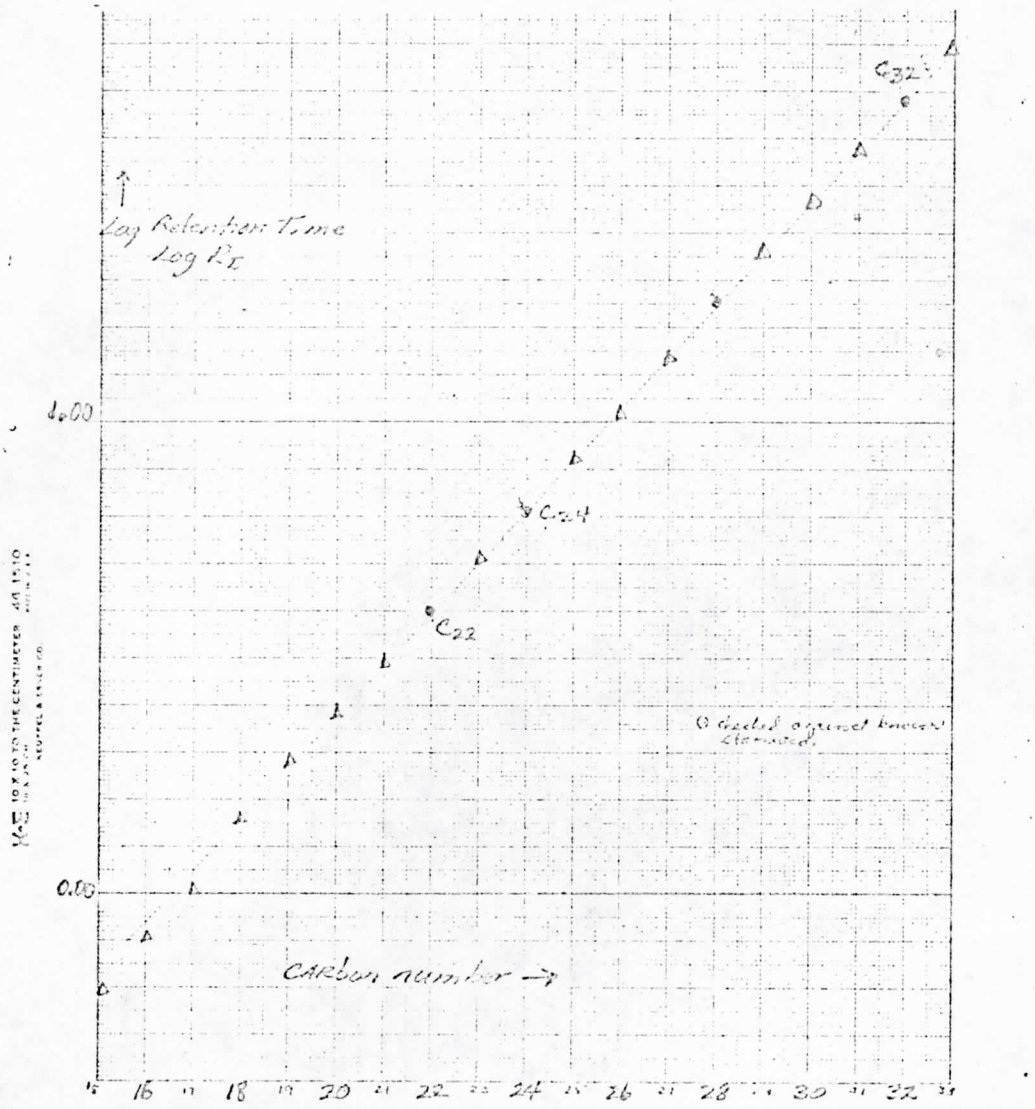
The flow rate for the SE-30 column was set at 30 $ml.min.^{-1}$ and for the Apiezon-L at 20 $ml.min.^{-1}$

Identification of specific peaks was accomplished by addition of a known n-alkane standard, C_{22}, C_{24}, C_{28}, C_{32} (Supelco, Inc. item number 7104) which resulted in an intensification of those peaks corresponding to the alkanes in the standard.

Further identification was accomplished by plotting the log of the retention time for a particular known peak vs. its carbon number. Such a plot resulted in a straight line relationship. The log of the retention times of the unknown peaks were then plotted and the carbon number of the alkane read directly from the plot. The use of this method is discussed in the literature.[31] Examples of this type of plotting is given in Figure 6 and Table 8.

The percentage of an individual n-alkane was reported relative to the area under an individual peak as com-

Figure 6



Plot of Log Retention Time vs. Carbon Number for Fern Alkane Fraction, Polystichum acrostichoides.

Table 8

Data For Figure 6

Assigned Carbon Number	Retention Time-- In centimeters	Log. Retention Time
15	.6	-0.778
16	.8	-0.903
17	1.1	0.041
18	1.45	0.168
19	1.90	0.279
20	2.38	0.377
21	3.10	0.491
22	3.95	0.597
23	5.05	0.703
24	6.50	0.813
25	8.30	0.919
26	10.25	1.011
27	13.75	1.138
28	18.05	1.256
29	22.75	1.357
30	29.20	1.465
31	37.30	1.572
32	47.55	1.677
33	60.85	1.784

Underlined figures are those belonging to the alkane Standard.

pared to the total area under all measured peaks. Areas were computed by the height times width-at-half height method.

Major unidentified peaks were reported according to their Kovat Retention Index Numbers.[32] This method assigns values to those peaks which occur between known peaks as a function of the retention times of the known peaks. Thus a peak occuring half way between C_{25} and C_{26} would be reported as having a Retention Index Number of 2550 since the assigned value of C_{25} and C_{26} are 2500 and 2600, respectively.

The use of the Kovat method thus permits the use of published retention data which is not related to the condition of the analysis (e.g. operating temperature or flow rate).

Infrared spectra were recorded on a Perkin-Elmer Infracord 137 by using NaCl cells vs. air.

BIBLIOGRAPHY

1. Kolattukudy, P. E., Science 159, 498 (1958)

2. Kolattukudy, P. E., Ann. Rev. Plant Physiol., 21, 163 (1970)

3. Eglinton, G., R. J. Hamilton and M. Martin-Smith, Phytochemistry, 1, 137 (1962)

4. Eglinton, G., Phytochemistry, 1, 89 (1962)

5. Eglinton, G. and R. J. Hamilton, "Chemical Plant Taxonomy", ed. by T. Swain, p.187. Acedemic Press, London and New York. (1963)

6. Douglas, A. G. and G. Eglinton, "Comparative Phytochemistry", ed. by T. Swain, p. 57. Acedemic Press, London and New York. (1966)

7. Berti, G. and F. Bottari, "Progress in Phytochemistry", ed. by L. Reinhold and Y. Liwschita, Vol. 1, p. 589. Interscience Publishers, New York. (1968)

8. Juniper, B. E., Endeavor, 18, 20 (1959)

9. Juniper, B. E., New Phytol., 58, 1 (1959)

10. Dewy, O. R., G. S. Hartley and J. W. G. MacLauchlan, Proc. Roy. Soc., B155, 532 (1962)

11. Martin, J. T. and R. F. Batt, Ann. Appl. Biol., 46, 375 (1959)

12. Juniper, R. E., New Scientist, 13, 75 (1962)

13. Mazliak, P., "Progress in Phytochemistry", ed. by L. Reinhold and Y. Liwschitz, Vol. 1, p. 49. Interscience Publishers, New York (1968)

14. McKenna, E. J. and R. E. Kallio, Ann. Rev. Microbiol., 19, 183 (1965)

15. Eglinton, G. and R. J. Hamilton, Science, 156, 1322 (1967)

16. Chibnall, A. C. and S. H. Piper, Biochem. J., 28, 2189 (1934)

17. Chibnall, A. C., H. E. El Mangouri and S. H. Piper, Biochem. J., 28, 2209 (1934)

18. Schuette, H. A. and H. Kahn, J. Amer. Chem. Soc., 30, 126 (1953)

19. Wanless, G. G., H. H. King, and J. J. Ritter, Biochem. J., 59, 684 (1955)

20. Carruthers, W. and R. A. W. Johnstone, Nature, Lond., 184, 1131 (1959)

21. Mold, J. D., R. K. Stevens, R. E. Means and J. M. Ruth, Biochemistry, 2, 605 (1963)

22. Mold, J. D., R. K. Stevens, R. E. Means, and J. M. Ruth, Biochemistry, 3, 1293 (1964)

23. Kuksish, A., Biochemistry, 3, 1086 (1964)

24. Sorensen, N. A., "Chemical Plant Taxonomy", ed. by T. Swain, p. 219, Acedemic Press, London and New York. (1963)

25. Bu'Lock, J. D., "Comparative Phytochemistry", ed. by T. Swain, p. 79. Acedemic Press, London and New York. (1966)

26. Mirov, N. T., J. Amer. Pharm. Ass., 41, 677 (1952)

27. Aplin, R. T., R. C. Cambie and P. S. Rutledge, Phytochemistry, 2, 205 (1963)

28. Mold, J. D., R. K. Stevens, R. E. Means and J. M. Ruth, Biochemistry, 2, 605 (1963)

29. Berti, G. and F. Bottari, "Progress in Phytochemistry", ed. by L. Reinhold and Y. Liwschitz, p. 590-623 Interscience Publishers, New York, 1968.

30. McNair, H. M. and E. J. Bonelli, "Basic Gas Chromatography", p.65, Consolidated Printers, Berkeley, Calif., (1969)

31. Ibid., p. 127

32. Ettre, L. S., Anal. Chem., 36(8), 31A (1964)

33. Garton, G. A., Nature, Lond., 187, 511 (1960)

34. Shorland, F. B., J. Sci. Ed. Agric., 12, 39 (1961)